Effect of Heating on Polymerization of Pig Skin Collagen Using Microbial Transglutaminase

Yuny Erwanto, Michio Muguruma*, Satoshi Kawahara, Takahiko Tsutsumi, Kazunori Katayama Kiyoshi Yamauchi, Toshiro Morishita[1], Yasuko Kai[2] and Shohei Watanabe[2]

Laboratory of Food Science and Nutrition, Department of Biochemistry and Applied Bioscience, Faculty of Agriculture Miyazaki University, 1-1, Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan

ABSTRACT : Polymerization of heated or unheated pig skin collagen using microbial transglutaminase (MTGase) was investigated. Pig skin collagen samples were heated or left unheated, then enzymatically polymerized with MTGase. SDS-PAGE was conducted to confirm the intermolecular polymer and the results showed similar bands between samples without MTGase and unheated samples with MTGase. The polymerized product of pig skin collagen was not formed in unheated samples, even when MTGase was added during incubation. Different results were obtained from samples heated at 80°C and 100°C for 2 min, whereas the SDS-PAGE pattern indicated that a polymer band was generated in both cases. The heat treatment successfully modified the native structure of collagen and also made collagen more reactable in the MTGase polymerization system. Scanning Electron Microscope (SEM) investigation of pig skin collagen showed a biopolymer structure through intermolecular collagen crosslinking, while there were no intermolecular crosslinks in samples not treated with MTGase. There were no significant differences in fibril diameter between treated samples and controls. These results suggest that heat treatment of native pig skin collagen enhanced the polymerization capability of MTGase. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 8 : 1204-1209)***

Key Words : Pig Skin Collagen, Microbial Transglutaminase, Biopolymer, Heat Treatment

INTRODUCTION

Collagen, the most abundant mammalian and avian protein, is a connective tissue constituent that is present in all tissues (Mccormick, 1999). Collagen is commonly used for sausage casing, biodegradable films, pharmaceuticals, leather products and biomaterial products owing to its ability to maintain consistent shape, suitable physical characteristics, and bioadaptability. Processing to improve the characteristics of collagen in foods is an important stage in the production of many high quality foodstuffs.

The incorporation of inter- or intramolecular covalent crosslinks into food proteins is thought to improve the physical and textural properties of many food products, such as tofu, boiled fish paste and sausage. Chemical and enzymatic procedures have been developed to introduce covalent crosslinks into proteins. Because of its natural origin and high specificity for substrates, the enzymatic procedure is more acceptable to regulatory authorities and consumers (Nonaka et al., 1989). Transglutaminase (TGase, protein-glutamine γ -glutamyl transferase, E.C. 2.3.2.13) is one of the enzymes used to catalyze formation of the glutamine-lysine bonds in many food proteins.

Our knowledge of transglutaminase has developed rapidly since Ca^{2+} -independent microbial TGase (MTGase) was discovered. This enzyme has been produced industrially on a large scale using variants of *Streptoverticillium mobaraense* (Ando et al., 1989) or *Streptoverticillium ladakanum* (Tsai et al., 1996). MTGase has previously been used to improve the functional properties of food proteins such as soybean, milk, beef, pork, chicken and fish proteins (Kang et al., 1994; Nonaka et al., 1992; Sakamoto et al., 1994; Jiang et al., 1998; Seguro et al., 1995; Yildirim and Hettiarachchy, 1998). MTGase is also able to covalently incorporate amino acids or peptides into proteins (Nonaka et al., 1996). Yildirim and Hettiarachchy (1998) used this enzyme to produce film by crosslinking whey protein and 11 S globulin. However, the use of Ca^{2+}-independent TGase to produce biopolymer by polymerizing pig skin collagen has not yet been studied, because the specific and compact structure of the pig skin collagen is difficult for TGase to polymerize.

The thermal denaturation of the collagen molecule is indicated by the collapse of the structure and consequent shrinkage of the fibers (Bailey and Light, 1989). We therefore hypothesized that the difficulty of polymerizing skin collagen could be resolved by heat treatment. Kang et al. (1994) was successful in enhancing the gelation of soy bean glycinin. Their results indicated that heat treatment increased the amount of lysine and glutamine residues on the surface of glycinin molecules. The objectives of the

* Address reprint request to Michio Muguruma. Tel: +81-985-58-7203, Fax: +81-985-58- 7203, E-mail: muguruma@cc.miyazaki-u.ac.jp

[1] Miyazaki Prefectural Food Research and Development Center, 16500-2, Higashi Kaminaka, Sadowara, Miyazaki Gun, Miyazaki 880-0303, Japan.

[2] Minami Nippon Meat Packers, Inc., 1193 Zaikoji, Hyuga Shi, Miyazaki 883-0021, Japan.

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present research were 1) to evaluate the effect of heat treatment on polymerization of pig skin collagen in an MTGase catalysis system; and 2) to investigate the structure of biopolymers made with MTGase by scanning electron microscopic observation.

MATERIALS AND METHODS

Materials

Transglutaminase (E.C. 2.3.2.13) from culture broth of *Streptoferticillium mobaraense* was obtained from Ajinomoto, Co., Ltd. (Tokyo, Japan). MTGase was dissolved in 5 mM NaN_3 and 20 mM NaCl, and the solution was filtrated through filter paper No. 5A (Advantec Toyo, Tokyo, Japan) after centrifugation at 8,000 rpm at 4°C for 20 min. Fresh pig skin was obtained from Minami Nippon Meat Packers, Inc. (Miyazaki, Japan). Samples were prepared from pig skin with an MKZA6-5 Super Masscolloider (Masuko Sangyo Co., Ltd., Saitama, Japan). The particle size was 0.1 to 0.3 mm (diameter), and the samples were stored at -80°C until use.

Preparation of pig skin protein polymer catalyzed with MTGase

Pig skin samples (2.5 g per tube) were placed into 50 ml conical tubes, supplemented with 12.5 ml of buffer solution (200 mM Na Phosphate, 20 mM NaCl and 4 mM NaN_3, pH 6.5) and 10 ml of distilled water, and mixed. Thus the final concentration of sample was 100 mg/ml (w/v wet basis). The samples were homogenized at 0°C in an ice bath, then divided into two groups, a heated (2 min at 80°C or 100°C) and an unheated group. The unheated samples (without and with MTGase; 0.5% w/w) were incubated at 37°C and 50°C for 24 h and 6 h, respectively, with shaking. The heated samples (with and without MTGase; 0.5% w/w) were incubated at 37°C for 24 h in the same instrument. After incubation, the enzyme reaction was terminated by addition of 60 mM NH_4Cl. The resulting products were centrifuged at 18,000 rpm for 30 min at 30°C, and then the supernatant and precipitate were collected individually. The precipitate was suspended in 10 ml of distilled water and diluted with 10 ml of 10% SDS. The samples in 5% SDS were incubated at 37°C overnight and centrifuged at 8,000 rpm for 30 min

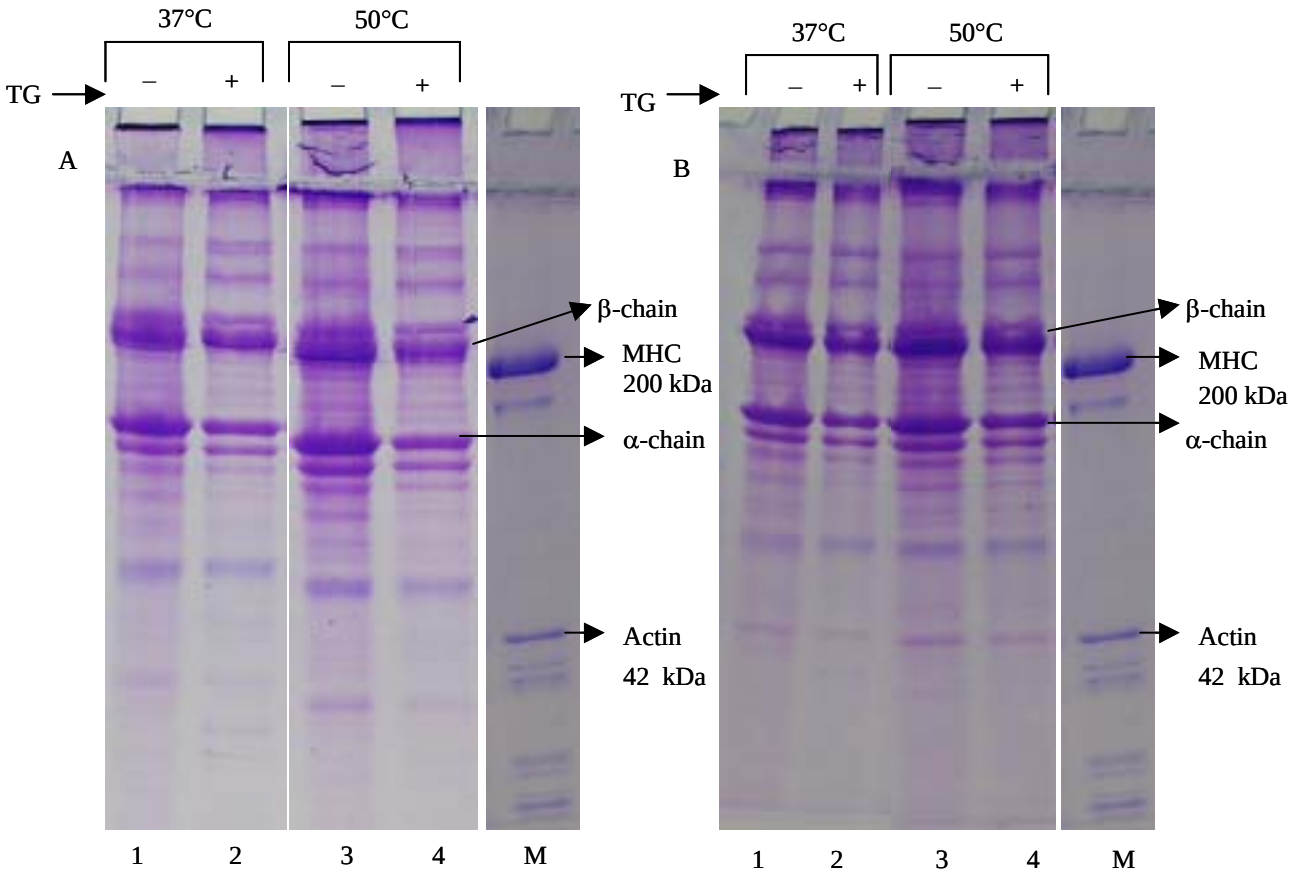

Figure 1. SDS-PAGE patterns of pig collagen polymer, with or without MTGase (0.5 % w/w). (A) Whole samples of polymer products; (B) Supernatant of polymer; samples were separated by centrifugation at 18,000 rpm for 30 min. Lane 1 and 2: samples were incubated at 37°C for 24 h; Lane 3 and 4: samples were incubated at 50°C for 6 h. M, myofibril standard; MHC, myosin heavy chain.

to separate the supernatant from the residue. The supernatant was dialyzed in a buffer (100 mM Na Phosphate, 20 mM NaCl, 2mM NaN_3 and 0.1% SDS, pH 6.5) overnight and transferred into a tube. Protein confirmation was confirmed by the biuret method using bovine serum albumin as a standard (Gornall et al., 1949).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition was performed using 5%-20% gradient acrylamide slab gel and an SDS-Tris-glycine discontinuous buffer system according to the method of Laemmli (1970). Prior to electrophoresis, the protein samples were heated at 95°C for 5 min in the presence of 25 mM Tris-HCl (pH6.8), 1% SDS, 20% glycerol, 0.01% bromophenol blue and 1% 2-mercaptoethanol. An aliquot of the sample solution was applied to gradient gel, and electrophoresis was performed at 20 mA/gel. After the electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue (CBB) in 50% methanol and 10% acetic acid, and destained in the same solvent without dye.

Scanning electron microscope (SEM)

A three-dimensional impression of the biopolymer structure was obtained using a scanning electron microscope. Samples of pig skin collagen after incubation with or without MTGase were collected by centrifugation at 12,000 rpm for 20 min. The precipitated samples were fixed in a buffer containing 3% glutaraldehyde and 0.12 M cacodylic acid sodium salt, pH 7.2, at 4°C, replacing 3 times every 2 h. Samples were washed with the same buffer without glutaraldehyde 4 times every 2 h at 4°C. After fixation with 1.33% osmium acid and 0.12 M cacodylic acid sodium salt, the samples were dehydrated by immersion in a series of ethanol mixtures, 50%, 70%, 80%, 90%, 95% and 100% and finally immersed in isoamyl acetate. After dehydration, critical point drying was performed in liquid CO_2 in a pressurized chamber. The dried samples were mounted on an aluminum stub and coated with gold by ion sputtering using an Hitachi E-1030. All samples were then observed and photographed using a scanning electron microscope (S-4100M; Hitachi Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Crosslinking of pig skin collagen by MTGase

The formation of collagen polymer through the intermolecular crosslinks was analyzed by SDS-PAGE. Figure 1A shows the SDS-PAGE patterns of whole pig skin collagen treated or untreated with MTGase as a crosslinking agent. The unheated samples showed similar band patterns, irrespective of whether or not they were treated with MTGase. There were no significant changes when the incubation temperature was increased to 50°C. SDS-PAGE analysis of the supernatant showed the same results as analysis of whole samples (figure 1B). In unheated samples, the bands were clearly similar between the samples treated and those not treated with MTGase. The structure of native pig skin collagen was not capable of incorporating the intermolecular bond of glutamine and lysine. When the samples were treated with preheating, there were bands changes, including disappearance of the band between 45 and 90 kDa (figure 2). The high molecular weight biopolymer bands, which could not enter the stacking gel of SDS-PAGE, were appeared with heat treatment at 100°C for 2 min (figure 3). This result indicated that the reaction of pig skin protein with MTGase was affected by heat treatment. Heating the sample at 100°C before the reaction with MTGase might have modified the native structure of pig skin collagen so that it was able to cleave. This result suggested that heating could modify the structure of pig skin collagen, as predicted by Bayle and Light (1989).

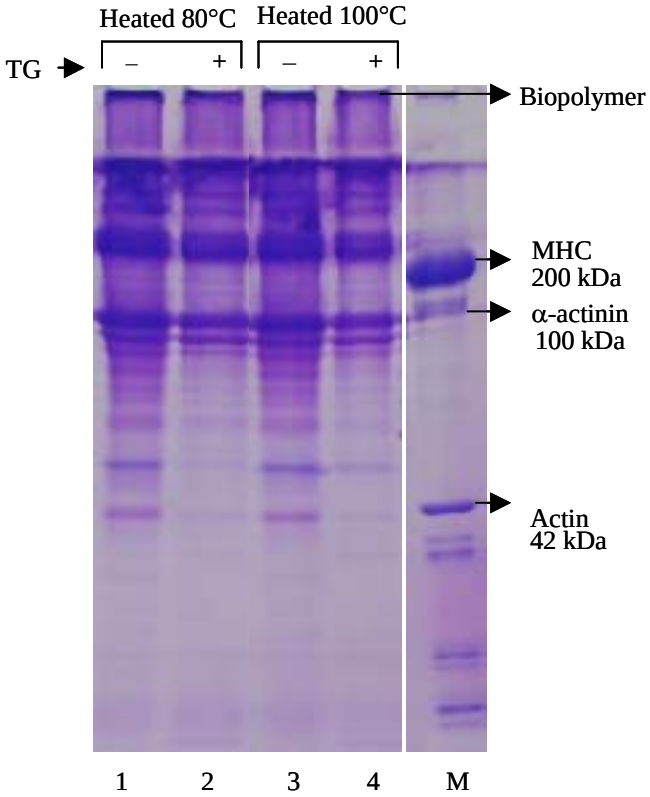

Figure 2. SDS-PAGE patterns of the samples reacted with or without MTGase (0.5% w/w). Before incubation samples were heated at 80°C (lane 1 to 2) and 100°C for 2 min (lane 3 to 4) followed by incubation at 37°C for 24 h with or without MTGase (0.5% w/w). M, myofibril standard; MHC, myosin heavy chain.

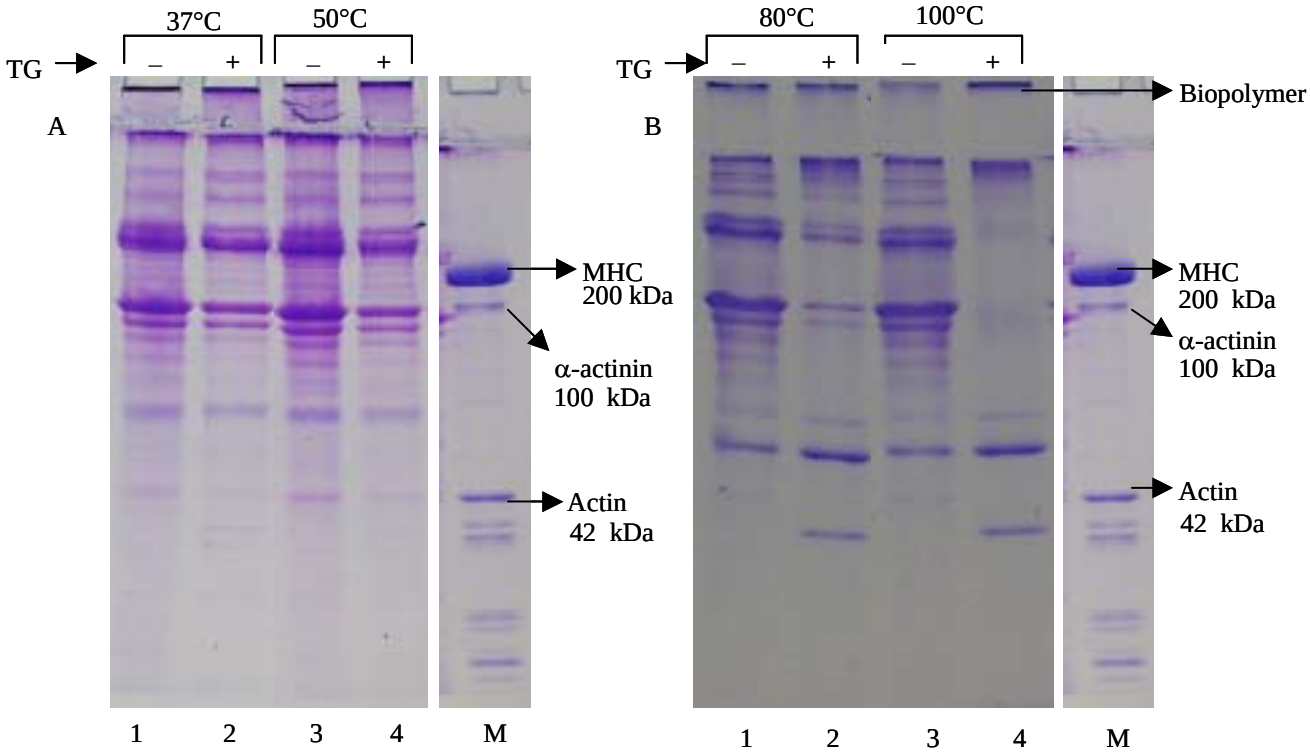

Figure 3. SDS-PAGE patterns of the samples reacted with or without MTGase (0.5 % w/w). (A) Unheated samples: (lane 1 to 2) samples were incubated at 37°C for 24 h; (lane 3 to 4) samples were incubated at 50°C for 6 h. (B) Samples were heated at 80°C (lane 1 to 2) and at 100°C for 2 min (lane 3 to 4) followed by incubation at 37°C for 24 h. M, myofibril standard; MHC, myosin heavy chain.

When heated in solutions, the rigid triple helical molecule of collagen produces a highly viscous solution, but at the denaturation temperature all structure is lost and there is a dramatic decrease in viscosity. Bayle and Light (1989) also stated that the denaturation of the triple helix of collagen has been shown to be a two-stage process with separation of the polypeptides as a first stage and the denaturation of their helical form as the second stage. The solution produced, known as gelatin, will generally contain not only free a α -chains but also dimer, trimer and higher molecular weight components. In this study, the transglutaminase reaction resulted in similar bands on the SDS-PAGE analysis of unheated samples. This may have been due to the compact structure of the triple helix of collagen. Another prediction of the phenomenon described above was caused by the little content of glutamine and lysine in collagen about 7.2% and 2.62%, respectively (Bienkiewicz, 1983), therefore the formation of ε-(γ -glutamyl)lysine bound was very difficult.

Previous investigations into transglutaminase as a polymerizing catalyst have involved preheating of such samples as egg white protein film (Lim et al., 1998), whey protein and 11 S globulin film (Yildirim and Hettiarchchy, 1998) and soy bean glycinin gel (Kang et al., 1994). To modify the structure of collagen, some researchers have conducted heat treatment before incubation with MTGase. In a study by Lim et al. (1998), heat treatment resulted in an SDS-PAGE band of protein biopolymer which intensified aggregate band and increased molecular weight of biopolymer. These studies suggested that the band of biopolymer collagen appeared in the sample that was heated at 100°C for 2 min followed by MTGase reaction. The unique band intensity indicated that MTGase was a suitable catalyst in the biopolymer production of collagen (figure 3). Heat treatment may cause unfolding of the pig skin collagen structure. It also noted that the basic subunits of pig skin collagen became susceptible to transglutaminase reaction by heat treatment and were polymerized.

Evaluation of collagen gel structure by SEM

The three-dimensional structure of pig skin collagen biopolymer formed by MTGase reaction is shown in figures 4 and 5. The triple helical structure consists of three polypeptide strands as a native structure of collagen without MTGase (figure 4A). Pig skin collagen crosslinked by MTGase showed unique structure results from intermolecular reaction (figure 4C and E). The crosslinked collagen molecule was more clearly shown in the preheated samples (figure 5B and D) compared with the unheated

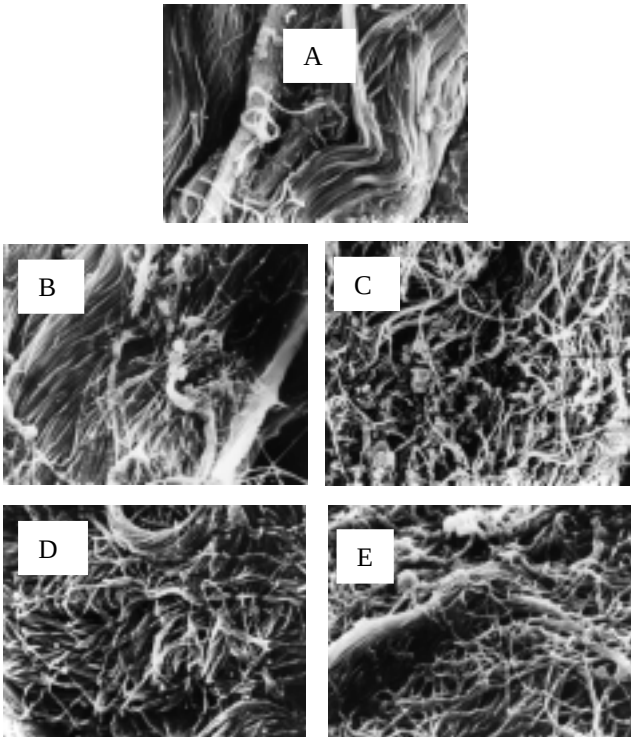

Figure 4. Scanning electron micrographs of unheated samples of pig skin collagen polymer with or without MTGase (0.5% w/w). Magnification is 4,500 X. (A) Native collagen; (B) Incubated at 37°C for 24 h without MTGase; (C) Incubated at 37°C for 24 h with MTGase; (D) Incubated at 50°C for 6 h without MTGase; (E) Incubated at 50°C for 6 h with MTGase. The calibration bar represents 16 μm.

samples (figure 4). The fibril diameter of the unheated sample without MTGase, the unheated sample with MTGase, the heated sample without MTGase, the heated sample with MTGase and native pig skin were 116.71 nm, 123.88 nm, 130.00 nm, 130.77 nm and 118.97 nm, respectively. The diameter of collagen for heated samples was slightly increased, but there were no differences on samples with or without MTGase addition. These results were consistent with previous research on pepsin-solubilized collagen polymerization (Takahashi et al., 1998).

Collagen has important functions in the textural change of various kinds of foods; however, the modification of collagen structure, especially in pig skin collagen, has not been studied. Sato et al. (1986) reported that the texture of cooked fish meat was affected somewhat by gelatin derived from the muscle collagen, based on the finding that cooked meats of species with high collagen content tended to show more elastic texture than those of species with relatively low collagen. The solubility of collagen in the heat process was affected by the structure and thickness of connective tissues. Generally, at temperatures above 70°C, severe denaturation occurred and resulted in pronounced shrinkage

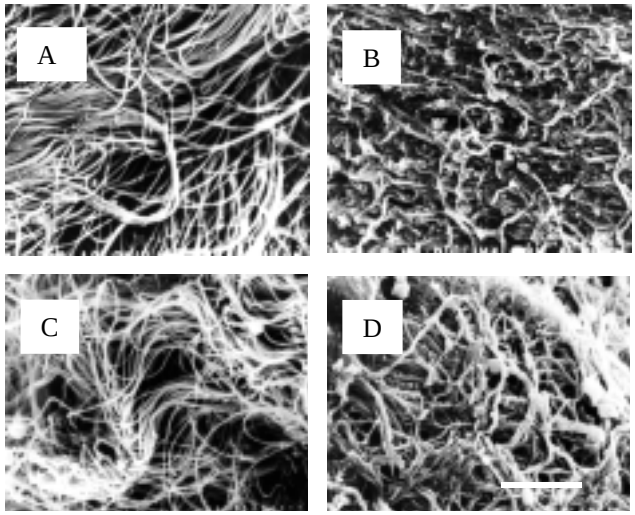

Figure 5. Scanning electron micrographs of heated samples of pig skin collagen incubated at 37°C for 24 h with or without MTGase (0.5% w/w). Magnification is 4,500 X. (A) Heated at 80°C for 2 min followed by incubation without MTGase; (B) Heated at 80°C for 2 min followed by incubation with MTGase; (C) Heated at 100°C for 2 min followed by incubation without MTGase; (D) Heated at 100°C for 2 min followed by incubation with MTGase. The calibration bar represents 16 μm.

of the meat sample. In the present study, the heat treatment was conducted to modify the structure of pig skin collagen, and the structure of protein became more soluble and more susceptible to MTGase reaction. The structure of intermolecular crosslinking formation by MTGase was not clearly shown for unheated samples reacted at 37°C and 50°C. However the increase of crosslinking apparently occurred in the samples that were preheated at 100°C for 2 min (Figure 5D). It has been reported that the thermal denaturation and aggregation of native egg protein causes major changes in protein structure (Lim et al., 1998). Our results suggested that under heating condition, the proteins were partially unfolded and more flexible than when in their native form (Mine et al., 1990). Proteins in this state are thought to be more susceptible to TGase attack (Matsumura et al., 1996).

Kang et al. (1994) revealed that a well-developed network structure could be formed by MTGase reaction. The crosslinking consisted of linear strands without clumps of aggregated proteins. In the present study, intermolecular reaction among single strands of collagen resulted in formation of a new structure of biopolymer collagen. A collagen biopolymer network was formed in the presence of MTGase as a catalyst, and its triple helical structure was different from that of native collagen. The modification of the collagen network structure may produce a biopolymer with new functional properties. Further studies must be conducted to clearly describe the new functional structure

of intermolecular collagen crosslinking and rheological properties.

CONCLUSIONS

Heat treatment of pig skin collagen could modify the structure of native collagen such that it becomes a reactable substrate of MTGase. Such treatment may be useful for maximizing the polymerization of pig skin proteins via MTGase. SEM investigation of the new structure of protein polymers of pig skin collagen indicated that its functional properties may have been different from those of the native structure. Further investigations will thus be needed to clarify the functional properties and the detailed conformation of pig skin collagen polymer products.

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1211